Transformation of potato (cv. Late Harvest) with the potato leafroll virus coat protein gene, and molecular analysis of transgenic lines

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Potato leafroll virus (PLRV) is one of the most destructive potato viruses in South Africa. In order to establish resistance against PLRV in the potato cultivar Late Harvest, the coat protein (CP) gene of a South African isolate of the virus was isolated, cloned into the plant transformation vector pBI121 and inserted into potatoes using Agrobacterium tumefaciens*-mediated transformation. Six plantlets, which appeared to be phenotypically normal, were regenerated from leaf disks under kanamycin selection. These lines were analysed for stable transgene insertion and expression. The presence of the PLRV CP,* uidA *(GUS) and* nptII *(kanamycin resistance) genes were shown using PCR. Southern blot analysis verified that the PLRV CP gene had been inserted into the genome of the transgenic potato lines. Coat protein could not be detected, but RNA dot blots demonstrated PLRV CP gene expression at the mRNA level. Expression of the* uidA *gene was investigated using a fluorometric assay, and it was observed that lines containing the PLRV CP gene in the antisense orientation exhibited GUS activity.*

Potato leafroll virus (PLRV), a member of the luteovirus group, is one of the most destructive viral diseases of potatoes in South Africa, reducing both quality and yield of the potato crop. PLRV is transmitted by aphids, principally *Myzus persicae* (Sulz.), and may be spread by infected seed potatoes.[1] In South Africa, spread of PLRV is controlled by the Seed Certification Scheme of 'Potatoes South Africa'. Because of the manner of transmission of the virus, control of PLRV by spraying with systemic insecticides has been only partially effective, and planting resistant cultivars is probably the most efficient method for controlling the disease.[2] As South African potato cultivars do not have host-mediated resistance to PLRV, molecular breeding for resistance to the virus is an attractive option, especially as the existing agronomic characteristics of the potato cultivar will not be altered. Genetically engineered resistance to a virus was first reported by Powel Abel *et al.*,[3] who observed a delay in symptom development when transgenic tobacco plants expressing the tobacco mosaic virus (TMV) coat protein gene were inoculated with TMV. It appeared that the coat protein interfered with uncoating of the infecting TMV particles.[4] Subsequently, the coat protein mediated resistance (CPMR) concept has been widely applied to other viruses and plant families.[5,6] Zaitlin[7] reported a count of CPMR studies involving 22 viruses from 14 virus groups. Resistance to PLRV in transgenic potatoes containing the PLRV CP gene has been reported.[8–10,12,13] This type of resistance to PLRV does not give the transgenic plant immunity to the virus, but appears to suppress virus titre. In some cases, the virus titre in the PLRV CP-transgenic plants was only 1% of that reached in susceptible, control plants,[9,12] as determined by ELISA analysis.

PLRV is a phloem-limited virus, with a single positive strand RNA genome and an isometrical particle morphology.[2] Viral particles consist of one main protein species, the coat protein, with a molecular mass of approximately 26 kDa as determined by SDS-PAGE.[14] The genome of PLRV consists of 5987 nucleotides and six open reading frames (ORF), arranged in two gene clusters separated by a small non-coding region.[15] Open reading frame 4 encodes the coat protein and was shown to be expressed via a 2.3 kb subgenomic RNA.[16]

In an attempt to obtain coat protein mediated resistance to PLRV in South African potato cultivars, the coat protein gene from a South African isolate of PLRV was isolated, cloned, sequenced and subcloned into a plant transformation vector. The PLRV CP gene was subsequently inserted into the potato cultivar Late Harvest (LH) using *Agrobacterium tumefaciens*-mediated transformation. Plantlets were regenerated and characterised.

Materials and methods

Isolation and cloning of the PLRV CP gene. A South African isolate of PLRV was purified according to the method described by Prins and Thompson.[17] RNA was extracted and purified from the viral isolate by the method of Burger and von Wechmar.[18] Synthesis of cDNA from RNA was carried out according to the protocol of Gubler and Hoffman[19] (cDNA synthesis system plus, Amersham). cDNA was made using the 'cDNA primer' (5′-GTCTACCTATTTGG-3′), which is complementary to nucleotides 4312 to 4325 of the PLRV genome.[15] PCR was used to isolate the PLRV CP gene from the cDNA using the following primers: PLRV CP-left (5′-GCAGGATCCTAATGAGTACGG-3′), containing a *BamH*I site and the start codon; and PLRV CP-right (5′-GCACTCGAGCTACCTATTTGG-3′), containing a *Sac*I site and the complement of the stop codon. These primers correspond to nucleotides 3691 to 3702 and the complement of nucleotides 4312 to 4323 of the PLRV genome, respectively.[15] Subcloning was carried out using standard molecular biology procedures.[20] Templates for DNA sequencing were prepared by Exonuclease III shortening of pSK-LR.[21] Dideoxy chain termination sequencing was followed.[22] The DNA sequences of the junction points between the 3′ end of the CP gene insert and the 5′ end of the *uidA* gene in the plasmids pB16-LR and pB13-LR were determined using the GUS sequencing primer (5′-TCACG-GGTTGGGGTTTCTAC-3′: Clontech).

Production of PLRV CP transgenic potato plants. Plasmids pB16-LR and pB13-LR were transformed into the *A. tumefaciens* strain LBA 4404 in a triparental mating process[23] with the helper plasmid pRK2013. *A. tumefaciens* colonies capable of growing on medium containing kanamycin (100 μg ml^{-1}) were selected for use in plant transformations.

An adaption of the leaf disk transformation protocol[24–26] was used. *In vitro* potato leaves (cv. Late Harvest) were pre-

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incubated on MS medium[27] containing 1.5 mg l^{-1} of the ethylene inhibiter silver thiosulphate (STS) for 48 h. Following this, the leaf pieces were immersed in the *A. tumefaciens* cell suspensions for 20 min, blotted on sterile filter paper and replaced onto the MS medium for 48 h. After co-cultivation, the potato leaves were placed onto regeneration medium (MS stocks, 20 g l^{-1}; sucrose, 2 mg l^{-1}; zeatin, 0.02 mg l^{-1}; NAA, 0.02 mg l^{-1}; GA3, 7.5 g l^{-1}; agar, pH 5.8) containing kanamycin (50 mg l^{-1}) and cefotaxime (250 mg l^{-1}). When regenerated shoots were approximately 5 mm in length, they were excised and rooted on MS medium containing 50 mg l^{-1} kanamycin and 250 mg l^{-1} cefotaxime. Plantlets approximately 5 cm in height were transferred to potting soil in the glasshouse.

PCR analysis. Small-scale potato genomic DNA extractions were made from leaves of plantlets grown *in vitro*.[28] Yields were 100–300 µg g^{-1} leaf tissue. PCR was performed using a Minicycler (MJ Research, Inc.) with 30 ng DNA template per 10 µl reaction volume under standard conditions [0.5 U *Taq* polymerase (Promega), 100 µM each dNTP and 0.5 µM of each primer]. Amplifications of the PLRV-CP, neomycin phosphotransferase II (*nptII*) and GUS (*uidA*) genes were carried out with the following primers and annealing temperatures: PLRV CP-left (65°C; only one primer required, see Results); *nptII*-left (60°C; 5′-GAGGCTATTCGGCTATGACT-G-3′), *nptII*-right (5′-ATCGGGAGCGGCGATACCGTA-3′); GUS-left (70°C; 5′-GGTGGGAAAGCGCGTTACAAG-3′), GUS-right (5′-GTTTACGCGTTGCTTCCGCCA-3′).

Southern hybridisation. Potato genomic DNA was isolated from leaves of glasshouse grown plants using a modification of the method of Dellaporta *et al.*[29] Two additional phenol:chloroform:isoamyl alcohol (25:24:1) steps were included. Yields of 40-60 µg DNA g^{-1} leaf were obtained. Aliquots of 20 µg potato genomic DNA were digested with either *Hind*III or *Eco*RI, and the products were resolved on a 0.75% agarose gel before transfer to a Magnacharge nylon membrane (MSI) by the method of Southern.[30] The DNA was fixed to the membrane by baking at 80°C for 1.5 h. The amount of the 15.5 kb positive control plasmids pB16-LR or pB13-LR to be the equivalent of 50 copies of the PLRV CP gene in 20 µg potato DNA was calculated as 1.9 ng (Fig. 3, lanes 9–12), based on the measurement that the constant amount of DNA present in a tetraploid potato cell is 8.4 pg.[31] The ^{32}P-labelled CP gene probe was prepared by random prime labelling of a 713 bp *Eco*RI-*Hind*III fragment from pSK-LR (Prime-It RmT Random Primer Labeling Kit, Stratagene). The fragment was purified from the gel prior to labelling using GELase (Epicentre Technologies). It was necessary to use the ^{32}P with the highest specific activity (6000 Ci $mmol^{-1}$; Amersham) to obtain the required sensitivity. Hybridisation was carried out for 16 h at 40°C with 20% formamide, Denhardt's solution and 100 µg ml^{-1} herring sperm DNA. The following 15 min washes were carried out: 2×(25°C, 2×SSC/0.1% SDS); 2×(65°C, 2×SSC/0.1% SDS); and 65°C, 0.1×SSC/0.1% SDS.[20] The part of the membrane containing the DNA size marker (Marker IV, Boeringher Mannheim) was cut off after DNA transfer and hybridised separately with ^{32}P-labelled Marker IV. Autoradiography was carried out by exposure of the membrane to X-ray film (Hyperfilm MP, Amersham) at –70°C (with intensifying screen) for 4–18 days. Exposure times for the marker were 1–3 hours.

RNA dot blot analysis. Total RNA from PLRV-CP transgenic plants was extracted using the method developed by Verwoerd *et al.*[32] Various amounts of total RNA were dotted onto a nylon membrane and the membrane was probed with the same double-stranded DNA probe used for Southern blot analysis.[20]

GUS assay. A GUS fluorometric assay was performed.[11] Crude leaf extracts were prepared in GUS extraction buffer [50 mM $NaPO_4$, 10 mM EDTA, 0.1% (w/v) Triton-X, 0.1% (v/v) Sarcosyl, 10 mM 2-mercaptoethanol]. The protein concentrations of the leaf extracts were determined using the Bradford macroassay method (Biorad). A standard curve was constructed using bovine serum albumin (BSA), which was used to determine the protein concentration of the leaf extracts. In order to measure the fluorescence of the samples, 40 µl of each leaf extract was added to 500 µl of GUS reaction buffer [GUS extraction buffer containing 1 mM 4-methyl umbelliferyl ß-D glucuronide (MUG)] and mixed thoroughly. The reaction mixture was incubated at 37°C, and 70 µl volumes were removed and added to stop buffer (0.2 M Na_2CO_3) at 5-min intervals until the reaction had run for 30 min. MUG is cleaved by the GUS enzyme to form the fluorescing product methyl umbelliferone (MU) and fluorescence of the stopped reactions was read on a fluorometer at an excitation wavelength of 360 nm. A MU standard curve was constructed by first diluting a 10 mM MU stock solution to make the following standard solutions: 0.0; 0.1; 0.2; 0.3; 0.5; 0.8; 1.0; 5.0; 8.0 and 10 µM MU. The fluorescence of these standards was determined and the values used to construct a standard curve. Using the BSA and MU standard graphs, the amount of MU produced in µmol MU per mg protein was calculated and plotted against time for each plant sample.

Results

Isolation of the PLRV CP gene. Potato leaves showing symptoms of PLRV infection were collected at the ARC-Roodeplaat Vegetable and Ornamental Plant Institute. PLRV particles were isolated and the viral RNA was extracted (data not shown). The PLRV CP gene was cloned in a two-step process. First, cDNA of the CP gene was made using the 'cDNA primer'; second, this cDNA served as a template for PCR amplification of the PLRV CP gene, which was cloned into the TA vector pCR1000 (Invitrogen) to produce plasmid pCR-LR. The CP gene was subcloned as a 713 bp *Eco*RI-*Hind*III fragment into the plasmid pBluescriptSK to produce pSK-LR. The DNA sequence of the insert in pSK-LR showed that the full length PLRV CP gene had been cloned corresponding to nucleotides 3693-4319.[15] The cloned product contained the PLRV CP-left primer sequence at the 5′ end and the cDNA primer sequence at the 3′ end as expected; however, a second copy of the PLRV CP-left primer sequence was found immediately downstream of the cDNA primer and the sequence corresponding to the PLRV CP-right primer was not present.

The CP of the South African PLRV isolate shared over 95% nucleotide and amino acid sequence identity to all isolates from other parts of the world (Table 1). In most cases, nucleotide differences were silent and amino acid differences were conservative. The sequence was most similar to that of the Canadian (15 nucleotide differences; five amino acid changes) and Australian isolates (four amino acid changes), compared to the European isolates (Table 1).

Preparation of transformation plasmids containing the PLRV CP gene. The PLRV CP gene was excised from pSK-LR as a 743 bp *Xba*I-*Alu*I fragment. This was inserted into the plant transformation vector pBI121 (Clontech), which had either been digested with *Xba*I-*Sma*I or *Xba*I, to produce plasmids pB16-LR and pB13-LR, respectively. pB16-LR contains the CP gene in the sense orientation with respect to the CaMV 35S promoter, whereas it is in the antisense orientation in pB13-LR (Fig. 1).

Table 1. Differences in CP sequence between the South African isolate* and isolates from other countries.

Country	Nucleotide differences	Amino acid changes
Canada[33]	15	5
Australia[34]	19	4
Cuba[35]	19	7
Poland[36]	19	7
Germany[37]	20	8
Scotland[15]	20	9
Italy[38]	21	9
Netherlands[13]	21	10

*The CP sequence has been deposited in the Genbank (accession # AF022782). Sequence alignments were done using the software Genepro (Version 6.10; Riverside Scientific Enterprises, WA).

This placed the CP genes in plant transcription cassettes upstream of the *uidA* gene/*nos* terminator, linked to the *nptII* gene, and situated between the right and left T-DNA borders, which renders them competent for transfer to the plant genome by *A. tumefaciens* (Fig. 1). It should be noted that the PLRV CP gene is not a translational fusion with the *uidA* gene in pB16-LR, since there is a stop codon at the end of the CP gene, which is separated by 64 nucleotides from the start codon of the *uidA* gene (Fig. 1). Additionally, the CP gene is in frame +1 and the *uidA* gene is in frame +3. DNA sequencing confirmed that the junction points were correct. For pB13-LR the *Xba*I site at the 5′ end of the CP gene had ligated to half of the *Xba*I site of pBI121, whereas the *Alu*I site at the 3′ end of the CP gene had ligated to the other half of the *Xba*I site of pBI121, which had unexpectedly been blunt-ended.

Production of PLRV CP transgenic potato plants. From 65 leaf disks that were inoculated with *A. tumefaciens* LBA 4404 (pB16-LR), three shoots were regenerated from callus produced at the cut edge of the leaf. Four shoots were also regenerated from 70 leaf disks inoculated with *A. tumefaciens* LBA 4404 (pB13-LR). All seven shoots rooted on medium containing 50 mg l^{-1} kanamycin. One plantlet containing the sense orientation of the PLRV CP gene appeared to be phenotypically abnormal, and was discarded. The remaining six LH transgenic plantlets appeared to be normal.

PCR analysis. Successful *A. tumefaciens*-mediated transformation results in transfer of the complete DNA fragment between the T-DNA borders into a plant cell, which subsequently regenerates into a plant. Three genes, namely PLRV CP, *nptII* and *uidA*, are expected to be present in the transgenic potato lines (Fig. 1). PCR analysis of genomic DNA with gene-specific primers was conducted. This showed that these genes were present in all six transgenic LH lines since amplification products of the expected size were obtained; 664 bp for PLRV CP (Fig. 2*A*, lanes 3–8); 600 bp for *nptII* (Fig. 2*B*, lanes 3–8);

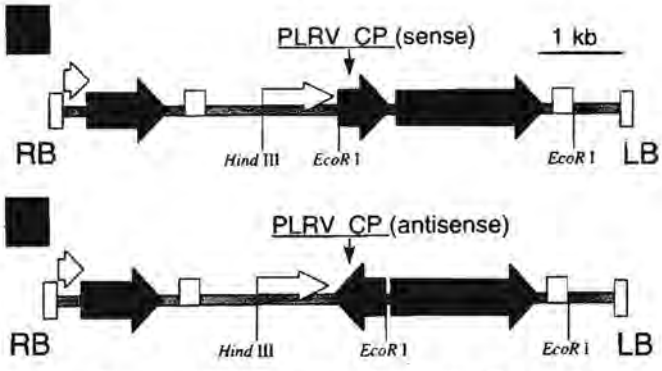

Fig. 1. Cartoon of T-DNA regions of plasmids pB16-LR (panel A) and pB13-LR (panel B). The *nptII*, PLRV CP and *uidA* genes are indicated by the name of their products: NPTII, PLRV CP (or antisense RNA) and GUS, respectively. The nopaline synthase (NOS) promoter (5′ to *nptII*) and the CaMV 35S promoter (5′ to the CP) are indicated by open arrows. The NOS terminators are indicated by open boxes. The T-DNA right and left borders are indicated by hatched boxes.

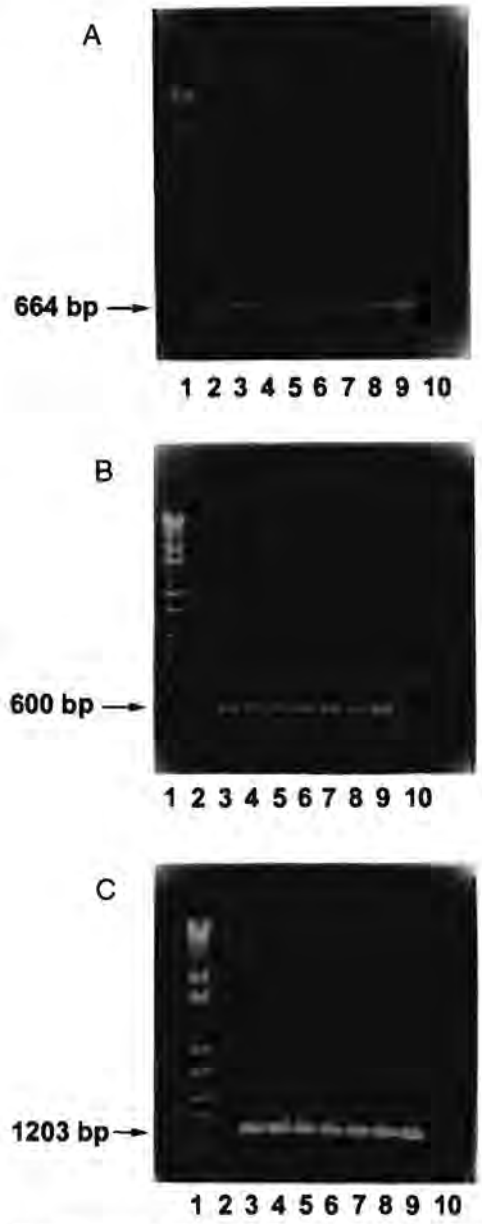

Fig. 2. PCR analysis of transgenic lines. Panel A (PLRV CP gene-specific primer); Panel B (*nptII*-specific primers); Panel C (*uidA*-specific primers). Genomic DNA isolated from untransformed LH, transgenic lines LH 13.1, LH 13.2, LH 13.3, LH 13.4, LH 16.2 and LH 16.3 was used as template DNA for PCR reactions shown in lanes 2–8, respectively. The order is identical in each panel. The positions of the expected 664 bp PLRV CP gene, 600 bp *nptII* and 1203 bp *uidA* PCR products are shown to the left of panels A, B and C, respectively. Lane 1 contains the λ Sty I DNA marker (19; 7.7; 6.2; 4.3; 3.5; 2.7; 1.9; 1.5; 0.9 and 0.4 kb). Lanes 8 and 9 contain PCR reactions with pB13-LR and no template, respectively.

Fig. 3. Autoradiograph of Southern blot of transgenic potato DNA digested with restriction enzymes. The probe was the radioactively labelled PLRV CP gene. Lanes 2–8 contained 20 µg of *EcoR*I digested genomic DNA from untransformed LH, transgenic lines LH 16.2 , LH 16.3, LH 13.1, LH 13.2, LH 13.3 and LH 13.4, respectively. Lanes 13–19 contained 20 µg of genomic DNA digested with *Hind*III from untransformed LH, transgenic lines LH 16.2 , LH 16.3, LH 13.1, LH 13.2, LH 13.3 and LH 13.4, respectively. Lanes 9 and 10 contained 5 µg of genomic DNA from the untransformed LH genomic DNA digested with *EcoR*I spiked with 1.9 ng of *EcoR*I digested pB13-LR and pB16-LR, respectively. Lanes 11 and 12 contained 5 µg of genomic DNA from the untransformed LH genomic DNA digested with *Hind*III spiked with 1.9 ng of *Hind*III digested pB13-LR and pB16-LR, respectively. Lane one contained DNA molecular marker IV (Boeringher Mannheim) probed separately with labelled marker. The sizes of marker fragments are shown on the left.

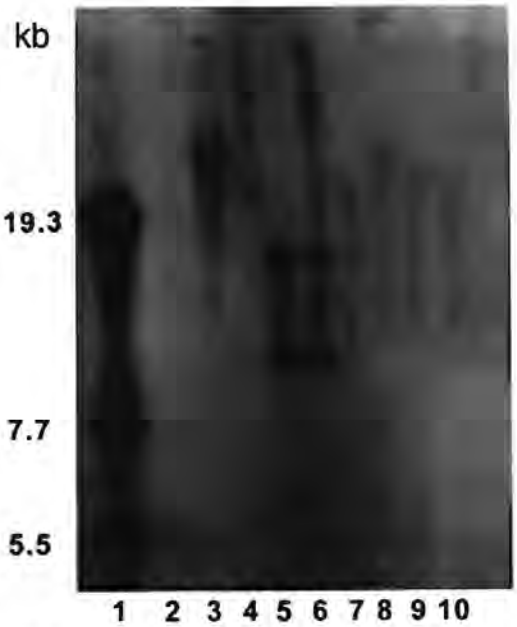

Fig. 4. Autoradiograph of Southern blot of uncut potato genomic DNA. The probe was the radioactively labelled PLRV CP gene. Lanes 2, 3, 4, 7, 8, 9, 10 contained 10 µg each of DNA from the untransformed LH, LH 16.2, LH 16.3, LH 13.1, LH 13.2, LH 13.3 and LH 13.4, respectively. Lanes 5 and 6 contained 10 µg of untransformed LH DNA spiked with the equivalent of 20 copies of uncut pB16-LR and pB13-LR, respectively. Lane 1 contained DNA molecular marker IV (Boeringher Mannheim) probed separately with labelled marker. The sizes of marker fragments are shown on the left.

and 1203 bp for *uidA* (Fig. 2*C*, lanes 3–8). These products were not amplified from genomic DNA of untransformed LH plants (lanes 2, Figs 2*A*, *B*, *C*) or for the negative control (DNA replaced with water; lanes 10, Figs 2*A*, *B*, *C*). The template in lanes 9 was the positive control, pB13-LR.

Southern blot analysis. Genomic DNA isolated from the transgenic potato lines was digested with restriction enzymes and probed with a radio-labelled PLRV CP gene probe. The probe hybridised to *Hind*III restriction fragments in all six transgenic lines (Fig. 3, lanes 14–19), whereas it did not hybridise to the untransformed LH DNA digested with *EcoR*I or *Hind*III (Fig. 3, lanes 2 and 13, respectively). These fragments were of different sizes to the *Hind*III products of pB13-LR and pB16-LR containing the CP gene (lanes 11 and 12, respectively), indicating that the hybridisation is from stable integration of the CP gene and not due to contaminating *A. tumefaciens* carrying the transformation plasmids. Since there is only one *Hind*III site between the T-DNA borders in the constructs used for transformation (Fig. 1), the number of *Hind*III fragments bound by the CP gene probe represent the number of insertion events of the PLRV-CP gene into the transgenic potato genomes and therefore represent the minimum copy numbers of the transgene. Minimum copy numbers of the transgene were three, two, two, one, one and one for lines LH 16.2, 16.3, 13.1, 13.2, 13.3 and 13.4, respectively. The hybridising fragment at 2041 bp (lanes 14–19) was not counted, since this was also present in the untransformed LH *Hind*III digest (lane 13), indicating that it was due to non-specific binding of the probe. The probe also hybridised to *EcoR*I restriction fragments in the six transgenic lines (Fig.3 , lanes 3–8). For the positive control plasmid (pB16-LR) and lines transformed with the sense construct (Fig. 3, lanes 10, 4 and 3, respectively) the probe hybridised only to a 3220 bp fragment, as expected, since the CP gene is flanked by *EcoR*I sites within the T-DNA borders (see Fig 1*A*). Therefore, irrespective of insertion site, these lines should yield an 3220 bp *EcoR*I fragment carrying the CP gene.

Additionally, it was shown that the transgene had been stably inserted into high molecular weight genomic DNA since positive hybridisation was seen with uncut DNA of all six transgenic lines (Fig. 4, lanes 3, 4, 7–10), whereas the probe did not bind to untransformed LH uncut DNA (Fig. 4, lane 2). The probe bound to uncut pB16-LR and pB13-LR plasmid DNA, but this was a lower molecular weight than the uncut genomic DNA (Fig. 4, lanes 5 and 6, respectively). Taken together, these results show clearly that the PLRV CP gene has been stably integrated in the six transgenic lines.

Analysis of transgene expression. Attempts were made to detect PLRV coat protein in the transgenic potato lines by immunoblotting and DAS-ELISA. No coat protein could be detected in these plants, even though non-transgenic leaf extracts (50 µg) spiked with PLRV did show antibody binding in immunoblots

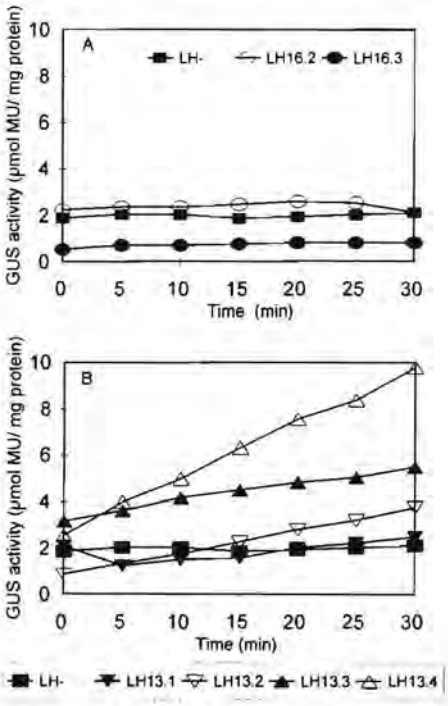

Fig. 5. GUS activity of transgenic lines. Panel A shows GUS activity (represented as µmol MU per mg plant material) over time of potato extracts from the lines containing the sense orientation of the PLRV CP gene (LH 16.2 and LH 16.3). Panel B shows GUS activity of lines with the antisense orientation of the PLRV CP gene (LH 13.1, LH 13.2, LH 13.3 and LH 13.4). The activity of the untransformed LH line (LH-) is given for reference on both panels.

(results not shown). The detection limit of antibody binding to PLRV was 10 ng in dot blots (results not shown).

A GUS fluorometric assay was used to detect GUS activity. The amount of MU produced in µmol per mg plant protein was calculated and plotted against time (Fig. 5*A*, *B*). There appeared to be a certain amount of background fluorescence present in LH potatoes, since most samples at time = 0 showed MU production of about 2 µmol per mg protein. No increase in MU production was seen in extracts from the untransformed LH plants (LH-), or plants containing the CP gene in the sense orientation (LH 16.2 and LH 16.3)(Fig. 5*A*). This was expected, since the *uidA* gene does not have its own promoter in the sense construct (Fig. 1). Unexpectedly, all four of the lines transformed with the antisense orientation of the PLRV CP gene (LH 13.1, LH 13.2, LH 13.3 and LH 13.4) showed increased MU production over time compared to LH- (Fig. 5*B*). Lines LH 13.3 and LH 13.4 had the greatest GUS activity (Fig. 5*B*).

Detection of PLRV CP mRNA transcripts. An RNA dot blot assay was carried out to determine if transcripts of the PLRV CP gene could be detected in the transgenic lines. Total RNA leaf extracts were probed with a double-stranded DNA probe (Fig. 6). Positive DNA-RNA hybridisation was visualised for all six of the transgenic lines, but not the untransformed LH samples, indicating that the CP transcript was made in all the lines. The positive control PLRV RNA samples were also bound by the CP probe; as expected, however, there appeared to be a quenching effect in the spiked sample (Fig. 6).

Discussion

The high CP sequence identity between all PLRV isolates worldwide is either a reflection of high selection pressure for a specific CP tertiary structure or may be accounted for by recent dissemination of PLRV from its centre of origin. This is likely since potato material was imported into South Africa before the 1940s with very few quarantine measures. It is interesting to note that the Australian isolate shows the closest amino acid identity to the South African isolate. These countries both have a warmer climate compared to those in the northern hemisphere.

The potato cultivar LH was successfully transformed with the CP gene from the South African isolate, and six visually normal transgenic lines were regenerated under kanamycin selection: two with the sense orientation of the PLRV CP and four with the antisense orientation. The presence of the *nptII*, *uidA* and PLRV CP genes were shown in all six lines using PCR analysis. Southern blot analysis confirmed that the PLRV CP gene had integrated into each potato genome. Line LH 16.2 has a minimum of three copies; however, it is likely to have extra copies in tandem, since the *EcoR*I and largest *Hind*III fragment hybridise with greater intensity than fragments in other lines (Fig. 3; lanes 3 and 14, respectively). The mRNA transcript of the PLRV CP gene could be detected for all six transgenic lines, but not the coat protein. This result correlates with those obtained by other researchers.[8,10,13,39] Interestingly, GUS activity was detected in transgenic plants containing the antisense orientation of the PLRV CP gene, but not in plants with the sense orientation. The plant transformation constructs pB16-LR and pB13-LR contain the PLRV CP gene positioned between the CaMV35S promoter and the *uidA* gene (Fig.1), so both genes will be transcribed on a single mRNA transcript. In pB16-LR, however, because of the presence of a translational stop codon (TAG) at the 3′ end of the PLRV CP gene, only the first gene (the PLRV CP gene) on the mRNA transcript will be translated into a protein, since the 80S ribosome moving along the mRNA will dissociate when it reaches the stop codon. Therefore, in the sense PLRV CP transgenic potato plants the PLRV CP gene could be translated but not the *uidA* gene (Fig. 5*A*). A possible explanation for GUS activity in the antisense PLRV CP transgenic potato plants could be that the *uidA* gene is recognised as the first gene on the mRNA, since the antisense CP gene does not have start or stop codons (Fig. 5*B*).

If this theory is correct, it may indicate that PLRV coat protein is produced in the transgenic plants, but at levels lower than our

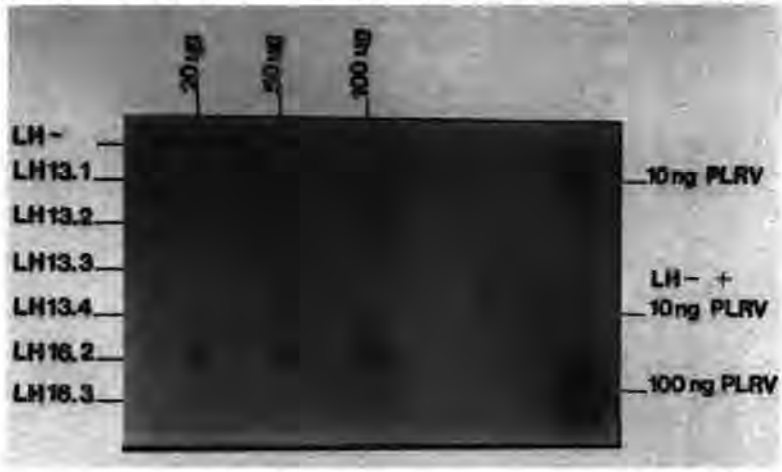

Fig. 6. Autoradiograph of RNA dot blot. Various amounts of total RNA (20 µg, 50 µg and 100 µg) extracted from potato lines untransformed LH (LH-), LH 13.1, LH 13.2, LH 13.3, LH 13.4, LH 16.2 and LH 16.3 were dotted onto a nylon membrane, and probed with a radioactively labelled PLRV CP probe. PLRV particles (10 ng and 100 ng) and 20 µg LH- total RNA spiked with 10 ng PLRV were dotted onto the right-hand side of the blot and served as positive controls.

Western blot detection limit of 0.02%. Kawchuk *et al.*[12] could detect coat protein at levels of approximately 0.01% of total phenol-soluble protein in transgenic 'Desiree' potato plants, but could not repeat this result for 'Russet Burbank' potato plants transformed with the PLRV CP gene. In addition, Barker *et al.*[8] detected coat protein sporadically and van der Wilk *et al.*[13] and Brown *et al.*[10] did not detect coat protein at all. In the experiments reported here, the PLRV coat protein could have been degraded in the cell or not produced at all. If the latter is the case, then resistance to PLRV in potatoes transformed with the PLRV CP could be RNA-mediated. RNA-mediated resistance may occur by RNA-RNA hybridisation. It can be speculated that the transgene mRNA produced by the plant may associate with the minus sense PLRV RNA replicative intermediate, thereby preventing viral replication. If this is the case, mRNA produced in transgenic plants containing the antisense orientation of the PLRV CP gene could associate with the positive-sense viral RNA, thereby also preventing viral replication. Resistance would be obtained in transgenic plants containing either orientation of the CP gene, and this has already been found to be the case.[8,39] We plan to test this for our transgenic potato lines by implementing glasshouse and field trials for PLRV resistance.

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